Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 337 (2005) 262 – 272

VIROLOGY

www.elsevier.com/locate/yviro

Antibody responses against wild-type yellow fever virus and the 17D vaccine strain: Characterization with human monoclonal antibody fragments and neutralization escape variants

Stephane Daffis[a], Roland E. Kontermann[b,1], Jehanara Korimbocus[c], Hervé Zeller[c], Hans-Dieter Klenk[a], Jan ter Meulen[a,*,2]

[a]*Institut für Virologie, Philipps-Universität, Robert-Koch-Strasse 17, 35037 Marburg, Germany*
[b]*Institut für Molekularbiologie und Tumorforschung, Philipps-Universität, Emil-Mannkopff-Str. 2, 35033 Marburg, Germany*
[c]*National Reference Center for Arboviruses and Viral Hemorrhagic Fevers, Lyon, France*

Received 20 January 2005; returned to author for revision 11 March 2005; accepted 25 April 2005

Abstract

Human monoclonal antibody fragments neutralizing wild-type and vaccine strains of yellow fever (YF) virus (genotypes West Africa I + II, East/Central Africa, 17D-204-WHO) were generated by repertoire cloning from YF patients. Analysis of virus escape variants identified amino acid (aa) 71 in domain II of the envelope glycoprotein (E) as the most critical residue for neutralization, with aa 153–155 in domain I contributing to the epitope. These data confirm the previous mapping of YFV neutralizing epitopes using mouse monoclonal antibodies but suggest that a conformational epitope could be formed by amino acids from domains I and II opposing each other in the dimeric form of the E protein. While the sera of the YF patients showed up to 10-fold reduced neutralizing activity against the 17D escape variants, sera from 17D vaccinees retained their neutralizing titers. Mutations in this major neutralizing epitope of YFV thus do not seem to carry the risk of immune escape in persons immunized with the YFV-17D vaccine.

Keywords: Yellow fever virus; Phage display; Immune library; Monoclonal antibody fragments; Envelope protein; Neutralizing epitope; Antibody response

Introduction

Yellow fever virus (YFV) is an important human pathogen, causing an estimated number of 200,000 clinically apparent infections and 30,000 deaths in tropical Africa and South America annually (Robertson et al., 1996). The spectrum of disease ranges from flu-like symptoms to fulminant hemorrhagic fever with multiorgan failure (Monath and Barrett, 2003). A live-attenuated vaccine (YFV strain 17D and related substrains), which induces humoral immunity against the currently known six genotypes of YFV, was developed in the 1930s through serial passage of wild-type YFV on different cell substrates (Monath, 2004). Neutralizing antibodies are predominantly directed against the envelope glycoprotein E of the virus (Brandriss et al., 1986; Gould et al., 1986; Pincus et al., 1992), which for other flaviviruses has been shown to govern the host cell receptor binding and endosomal pH-dependent membrane fusion (Lindenbach and Rice, 2001). Discrete neutralizing epitopes involving the envelope

* Corresponding author.
E-mail addresses: daffis@mailer.uni-marburg.de (S. Daffis), Roland.Kontermann@izi.uni-stuttgart.de (R.E. Kontermann), zeller@cervi-lyon.inserm.fr (H. Zeller), klenk@mailer.uni-marburg.de (H.-D. Klenk), j.termeulen@crucell.com (J. ter Meulen).
[1] Present address: Institut für Zellbiologie und Immunologie, Universität Stuttgart, Allmandring 31, Stuttgart, Germany.
[2] Present address: Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.04.031

protein amino acids (aa) E-71, E-72, E-155, E-158, E-173, E-240, E-305 and E-325 have been mapped on the monomeric E protein using mouse monoclonal antibodies, but no molecular studies of human antibody responses to YFV have been performed to date (Lobigs et al., 1987; Ryman et al., 1997a, 1997b, 1998).

The 17D vaccine is regarded as very safe and highly effective; however, serious and potentially fatal side-effects have recently been reported mainly in elderly persons (Kitchener, 2004; Lawrence et al., 2004; Martin et al., 2001). Because the currently used 17D vaccines essentially constitute quasi-species of closely related viral variants and the neutralizing epitopes of YFV have not been mapped for humans, it cannot be ruled out that naturally occurring neutralization escape variants might be present in 17D vaccine preparations (Pugachev et al., 2002). To gain insight into this problem, we mapped the neutralizing epitopes of the YFV-E protein using human monoclonal antibody fragments derived from yellow fever patients and generated neutralization escape variants of the 17D-204 vaccine strain. The rationale for screening patient immune libraries with the vaccine virus (as opposed to the homologous wild-type virus) was to identify yellow fever type-specific epitopes; furthermore, 17D yellow fever virus antigen can be handled without chemical or physical inactivation under biosafety level 2 conditions, thus sensitive conformational epitopes are not denatured.

Results

Generation of antibody phage display libraries and selection of antibody fragments reactive with YFV

Two antibody-phage libraries (size 8×10^6 and 7×10^7 clones, respectively; >75% correct single-chain antibody inserts) were constructed by repertoire cloning from yellow fever patients, subsequently pooled and panned with glycerol-purified non-inactivated YFV-17D virions. 50 monoclonal phage clones from selection rounds 3 and 4 showed specific binding to the YFV antigen in ELISA (data not shown). BstN1 fingerprinting revealed six distinct restriction patterns (data not shown); all clones representative of each pattern showed strong binding to YFV but not to BSA (data not shown). Sequencing of the single-chain antibodies (scFv) revealed usage of only two different V_H genes, most closely related to germline genes V_H4-59 and V_H3-11, and three different V_L genes, related to germline genes VκL-12, VL1-19 and VL2-14 (Fig. 1). All complementarity determining regions (CDR) of the V_H domains of scFv-5A, 7A and R3(27) were identical; the few differences observed in framework 1 (FR1) and FR4 are probably due to the usage of degenerated primers for PCR amplification. The V_L domains of these three antibodies were similar, differing mainly in the CDR2 and CDR3 regions. The V_H domains of scFv-1A, 2A and R3(9) were also identical, but only two of the V_L domains were similar, with substitutions

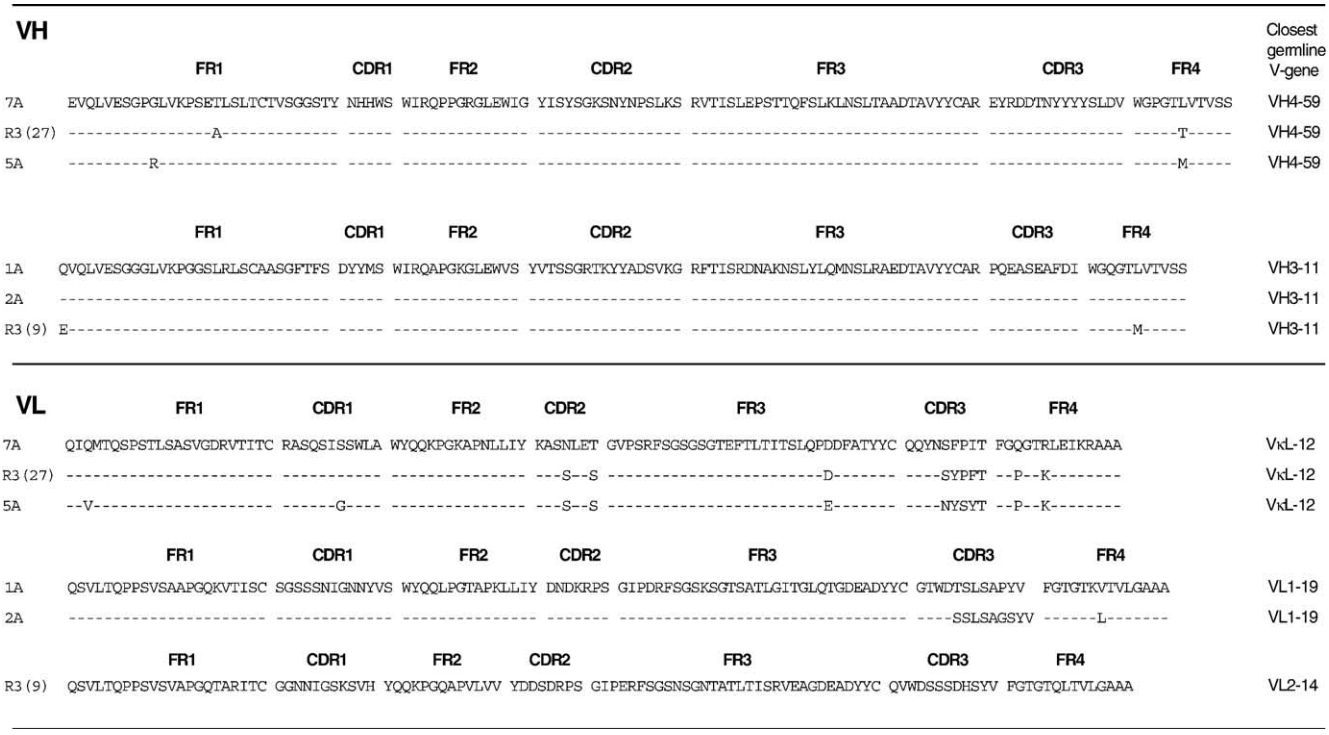

Fig. 1. Alignment of the variable heavy (V_H) and the variable light chains (V_L) of scFv reactive with YFV in ELISA. Framework regions (FR) and complementarity-determining regions (CDR) are indicated in bold. The closest germline V-gene for each chain was identified using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/).

located in CDR2 and CDR3 (GenBank accession nos. for the scFv: AY661699, AY661700,AY661701, AY661702, AY661703 and AY661704).

Genotyping of wild-type YFV strains used in the study

The strains Central African Republic (CAR) 1986 and Ethiopia 1961 were found to belong to the genotype East Africa/Central Africa (EA/CA) and the strain Senegal 1990 to the genotype West Africa II (WAII). The Nigeria 1987 and Ghana 1927 (Asibi) strains have been previously identified by Mutebi et al. (2001) as belonging to West Africa I (WA I) and West Africa II (WA II), respectively (GenBank accession nos. for the E sequences: AY839634, AY839635 and AY839636). In 2000, a single strain of YFV caused the yellow fever epidemic in Guinea, West Africa, during which the two patients of this study were identified (ter Meulen et al., 2004). The envelope sequence of this YFV was identical to that of the Asibi strain, with the exception of an N450S mutation (AY502949).

Soluble scFv neutralize YFV-Asibi, YFV-17D and wild-type YFV strains of genotypes West Africa I and II and East/Central Africa

As shown in Fig. 2, the three closely related scFv-5A, 7A and R3(27) showed 50% and 100% neutralizing activity in plaque reduction neutralization assay (PRNT) against YFV strains 17D-204-WHO (A) and Asibi (B) at concentrations of approximately 1 μg/ml and 10 μg/ml, respectively, whereas the three other scFv did not neutralize at any concentration tested. YFV type-specific mAbs 6538 and 6330 directed against the E protein were used as controls (Gelderblom et al., 1985). An irrelevant scFv isolated from the same library was included as negative control. Fig. 3 shows that scFv-5A, 7A and R3(27) neutralized wild-type YFV strains of genotypes West Africa I (Nigeria 1987) and II (Asibi) and East/Central Africa (CAR 1986, Ethiopia 1961) with comparable efficiency in a PRNT on PS cells. The concentration of scFv required for 90% plaque reduction ranged from 0.1 to 3.0 μg/ml of antibody fragment, with scFv-5A showing the highest potency. Interestingly, the strain Senegal 1990 was neutralized four to 10-fold less efficiently than all other strains. This strain was found to differ from Asibi by an N153K mutation and three additional mutations (T7A, A54V, N249D) in the envelope, two of which are located in the vicinity of the amino acids forming the neutralizing epitope (see below, and Fig. 7). Of the second group of closely related antibody fragments, only scFv-R3(9) showed some activity, neutralizing the YFV strain Nigeria 87 to maximally 60%. The envelope gene of this strain is identical to Asibi, with the exception of the mutations F426L and V430I. No scFv neutralized a West Nile virus strain isolated in France in 2000.

All neutralizing scFv bind to the same or closely related conformational epitope on the E protein

To identify the proteins recognized by the neutralizing scFv, an immunoprecipitation assay with radiolabeled, purified YFV-17D virions was performed. All scFv as well as the two envelope-specific mouse mAbs were found to precipitate a 55-kDa protein, corresponding to the molecular weight of the envelope glycoprotein (Fig. 4B). MAb 6330 but not mAb 6538 and none of the scFv reacted with a band of the same molecular weight in Western blot (Fig. 4A). This 55-kDa band was not precipitated by an irrelevant scFv from the same library

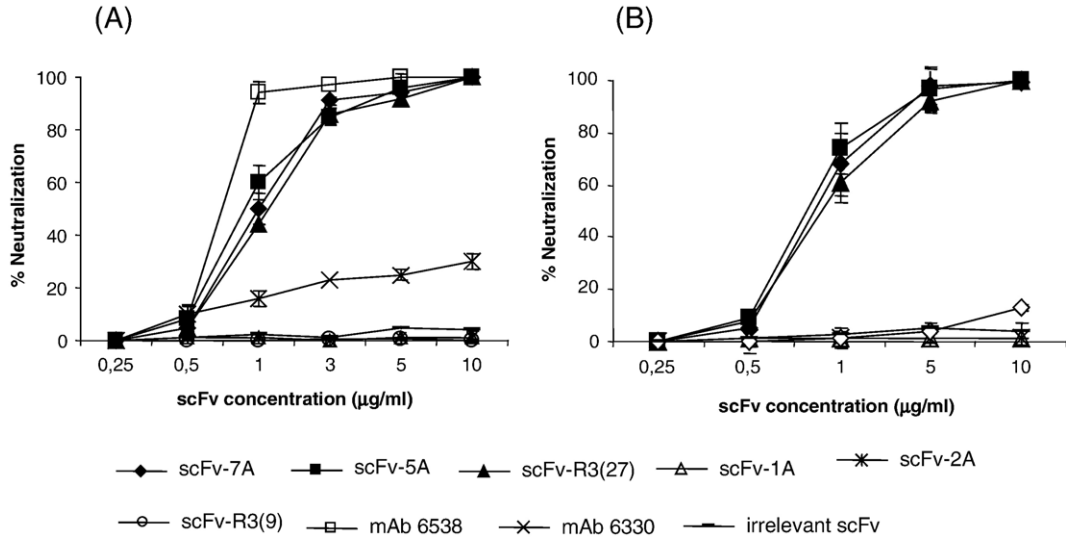

Fig. 2. Neutralization of the 17D-204-WHO (A) and the Asibi (B) strain of YFV by scFv in plaque reduction neutralization test (PRNT) using Vero cells. YFV-specific mAbs 6538 and 6330 were used as positive controls. As a negative control, an irrelevant scFv from the same library was used.

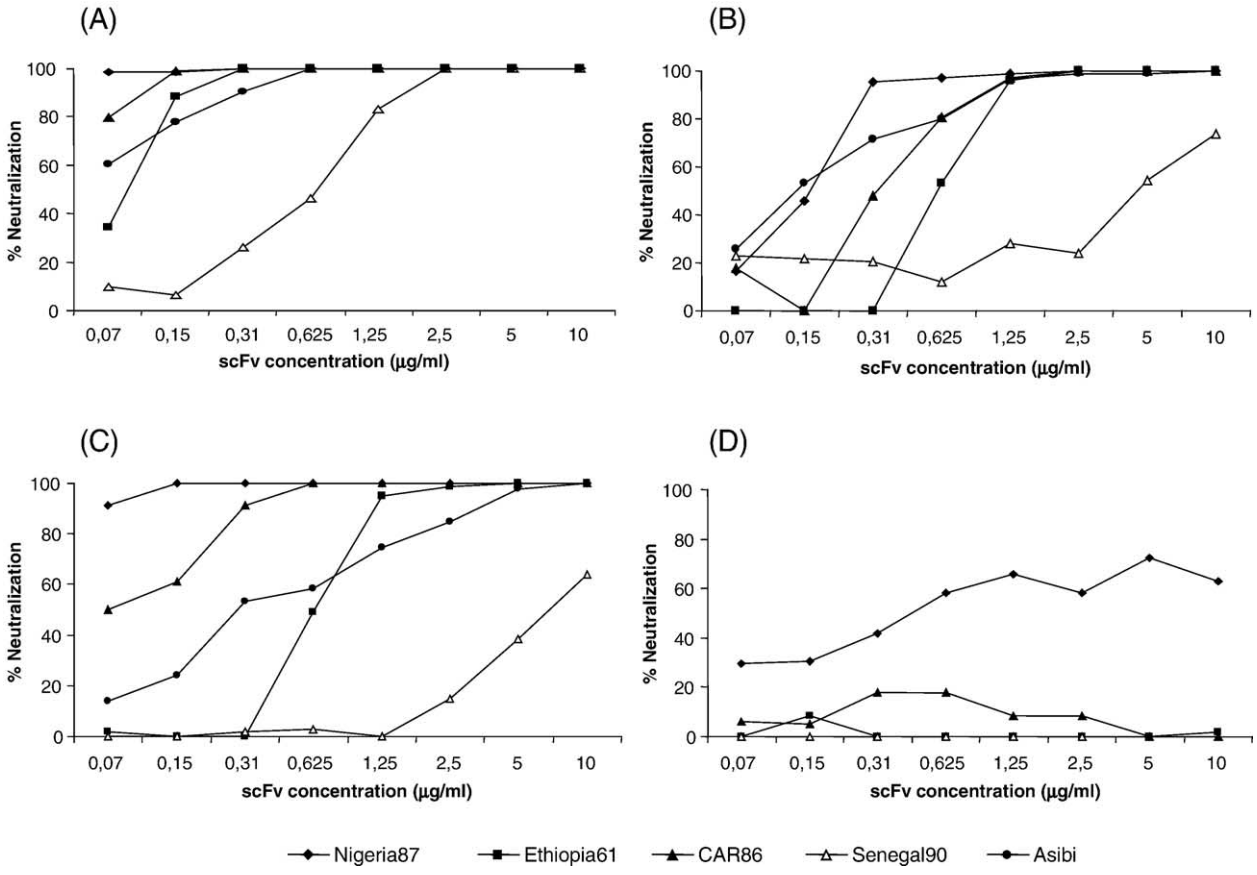

Fig. 3. Neutralization of wild-type strains of YFV by scFv-5A (A), scFv-7A (B), scFv-R3(27) (C) and scFv-R3(9) (D) in PRNT using PS cells. As a negative control, an irrelevant scFv from the same library was used.

included as negative control nor by the anti-His tag antibody alone. In a competition ELISA using biotinylated scFv-7A, this antibody fragment competed for binding to YFV-17D virions with the other neutralizing antibody fragments 5A, R3(27) and R3(9), but not with the non-neutralizing scFv-1A and 2A (Fig. 5). It can thus be concluded that all neutralizing scFv bind to the same or closely related conformational epitope on the envelope protein.

The amino acids critical for neutralization are located in domains I and II of the E protein and oppose each other in the dimeric form of the molecule

Four YFV-17D-204-WHO variants resistant to neutralization with scFv-7A were generated (Fig. 6). Mutants 7A-14 and 7A-18 showed low neutralization resistance (75% neutralization) and 7A-10 intermediate resistance (50% neutralization). The fourth mutant, 7A-12, was almost

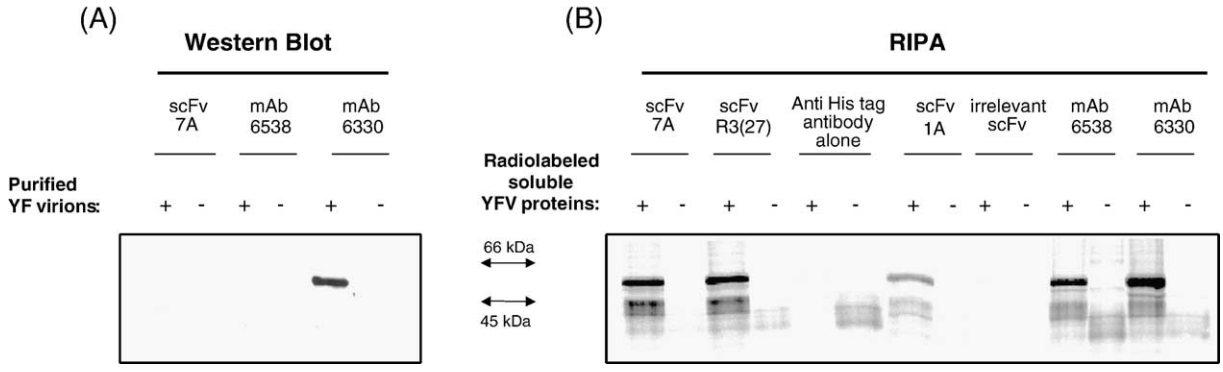

Fig. 4. Reactivity of scFv with YF-E protein. (A) Analysis of scFv reactivity in Western Blot. All scFvs and two mAbs were tested separately for their reactivity against purified YFV virions. (B) Immunoprecipitation of radiolabeled soluble YF viral proteins. Radiolabeled soluble YFV-E proteins were immunoprecipitated with each scFV. YFV-E-specific mAbs 6538 and 6330 were used as positive controls. As negative controls, an irrelevant scFv from the same library and an anti His-tag antibody were used.

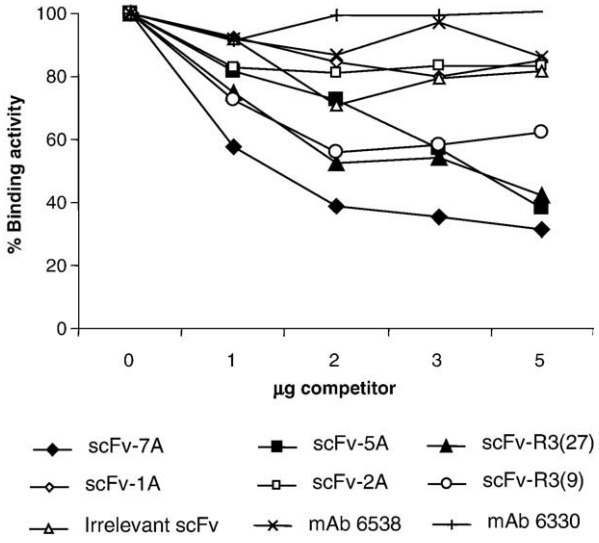

Fig. 5. Competition of scFv or E-specific mAbs for binding to YFV 17D-204-WHO virions using biotinylated scFv-7A. Unlabeled scFv or mAbs were used as competitor.

completely resistant to neutralization by scFv-7A (max. 5% neutralization). The sequence of the complete membrane and envelope genes of all escape variants and the parental 17D strain revealed mutations only in the E protein gene (Table 1). A total of four amino acid changes were identified: N71K in domain II (escape variant 7A-12), T153A in domain I (variant 7A-18), T153A and T154A (variant 7A-14) and D155G (variant 7A-10). The mutation at E-71 conferred the highest neutralization resistance.

The published crystal structure of the closely related dengue virus type 2 glycoprotein E was used to model the structure of the YFV-E protein and locate the escape mutations. As shown in Fig. 7, all mutations are exposed on the surface of the YFV E protein; however, because of spatial separation, domains I and II are unlikely to participate in forming an epitope in the monomeric E protein. However, in the homodimeric, antiparallel orientation which the molecules are predicted to adopt on the surface of the virion, the domain I and II from two E proteins are closely opposing each other (distance 15–20 Å). It is thus possible that the neutralizing epitope is formed by two E proteins, which each contribute a different domain.

YFV-17D escape variants generated with scFv-7A are efficiently neutralized by sera of YFV-17D vaccinees

To evaluate the importance of mutations in aa E-71 and E-155 with respect to neutralization escape of YFV-17D virus variants in human polyclonal sera, microneutralization assays were performed using sera from West African yellow fever patients and sera of travelers who had received routine YFV-17D immunization. Fig. 8 shows the 50% end-point dilution (NT_{50}) of the sera of two yellow fever patients (sera A) and eight 17D vaccinees (sera B) with YFV-17D-204-WHO and the derived neutralization resistant variants 7A-12 (N71K) and 7A-10 (D155G). Both sera of the yellow fever patients neutralized 7A-12 up to 10-fold less effectively compared to the parental 17D-204-WHO strain, whereas the sera of the immunized travelers showed unchanged or only slightly reduced neutralizing activity against 7A-12. Paradoxically, the neutralizing titers of most sera against the escape variant 7A-10 were significantly higher (up to 10-fold) compared with their titers against the parental 17D virus.

Discussion

Yellow fever virus neutralizing antibodies are predominantly directed against the envelope glycoprotein E, the crystal structure of which has been solved for the related flavivirus tick borne encephalitis (TBE) virus and dengue virus (Kuhn et al., 2002; Rey et al., 1995). Because of a high degree of sequence similarity and conservation of all cysteine residues, it is assumed that the E proteins of all flaviviruses share a common three-dimensional structure. The ectodomain of the E protein folds into three distinct domains, a central domain (I), a dimerization and fusion domain (II) and an IgG-like domain responsible for interaction with the unknown cellular receptor (III). Mapping of neutralizing epitopes on the YFV-E protein using mouse monoclonal antibodies has previously revealed critical amino acid residues in domain II (aa E-71/72, E-173), domain III (E-305/325) and domain I (aa E-155/158) (Lobigs et al., 1987; Ryman et al., 1997a, 1997b, 1998).

To define the human immune response to YFV on the epitope level, we generated human monoclonal antibody

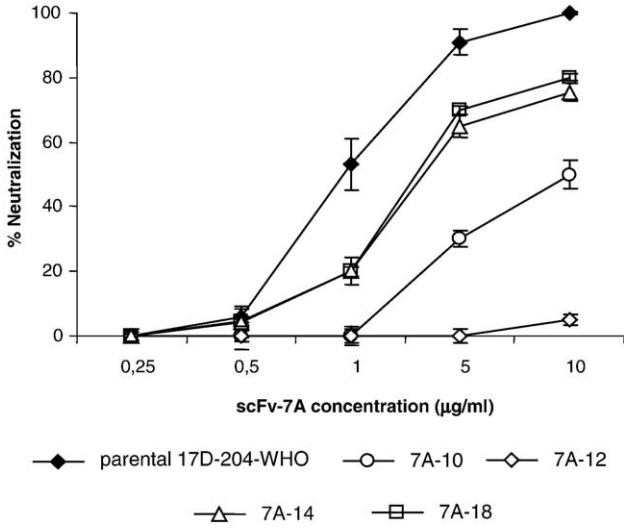

Fig. 6. Neutralization sensitivity of YFV-17D escape mutants to scFv-7A. The scFv-7A-neutralization resistance of four escape mutants (7A-10, 7A-12, 7A-14 and 7A-18) which were selected in the presence of subneutralizing concentrations of scFv-7A was tested in PRNT using various concentrations of scFv-7A. As a positive control, the parental YFV-17D-204-WHO virus was used.

Table 1
Nucleotide and amino acid differences in the E protein between YFV-17D-204-WHO virus and scFv-7A escape mutants

Escape variants	nt changes[a]		aa changes[a]		E protein domain	Percentage of neutralization by scFv 7A[b]	Published aa[c]
7A-14	nt 1430	A-G	E153	T-A	I	75%	T[d], N[e], K[f]
	nt 1433	A-G	E154	T-A	I		T[g], A[h]
7A-10	nt 1437	A-G	E155	D-G	I	50%	D[i], S[j], G[k], A[l]
7A-18	nt 1430	A-G	E153	T-A	I	75%	see above
7A-12	nt 1186	T-G	E71	N-K	II	5%	D[m], K[n], H[o], Y[p]

[a] Compared to the parental 17D-204-WHO strain.
[b] Plaque reduction neutralization tests performed with 10 μg/ml purified soluble single-chain Fv fragments.
[c] Amino acid published for positions E-71, E-153, E154 and E-155 in wild-type YFV, vaccine strains, escape variants or adapted strains.
[d] In vaccine strains 17D-204-WHO (Jennings et al., 1993), 17D-213-WHO (GenBank accession #AAC54268) and 17D-204-USA (#AAC35899).
[e] In vaccine strains 17D-204-Pasteur (#P19901), 17D-204-ATCC, 17DD from Brazil (#U17066) and Senegal (Jennings et al., 1993) and in all wild-type YFV strains.
[f] In the French neurotropic virus (#U21055) and a YFV strain isolated from mosquitoes in Trinidad (#U23568).
[g] In all YFV strains except footnote h.
[h] In three South American YFV strains isolated from humans (#U23566, #U23565 and #P29165).
[i] In all YFV strains except footnotes j, k, l.
[j] In 17DD from Brazil and Senegal.
[k] In a 17D-204-WHO escape variant (Ryman et al., 1997a) and in a fatal case of post-vaccinal encephalitis (Jennings et al., 1994).
[l] In Asibi virus adapted to Hela-cells (Dunster et al., 1999) and to Syrian hamster (McArthur et al., 2003).
[m] In a YFV strain isolated from mosquitoes in Trinidad (same isolate as in footnote f).
[n] In 17D-204-ATCC escape variants (Lobigs et al., 1987).
[o] In 17D-204-ATCC escape variants (Lobigs et al., 1987).
[p] In 17D-204-ATCC escape variants (Lobigs et al., 1987).

fragments through repertoire cloning from two West African yellow fever patients and screened the libraries with a vaccine strain of YFV. Three of the four neutralizing single-chain antibody fragments isolated had almost identical V_H and very similar V_L regions and all four competed for the same conformational epitope on the envelope glycoprotein. This epitope was mapped to a critical amino acid in domain II (E-71), with several amino acids in domain I contributing to a lesser extent to neutralization (E-153, E-154, E-155). No mutations were identified in domain III, which is responsible for attachment of the virus to the cellular receptor. Based on modeling of the sequence of the yellow fever E protein on the structure of the dengue virus type 2 envelope protein (Fig. 7), aa E-71 and E-155 are spatially separated by 65–70 Å in the monomeric protein. This distance is far beyond the surface area which the V_H and V_L chain of a single-chain antibody fragment can cover, ranging typically from 15 Å × 20 Å to 20 Å × 30 Å (Davies and Padlan, 1990). However, in the homodimeric form of the molecule, domains I and II of opposing monomers are predicted to be in close vicinity with a separating distance of 15–20 Å between aa E-71 and E-155 (Fig. 7). Furthermore, we identified naturally occurring mutations in close vicinity to E-71 (i.e., E-249) and E-153 (i.e., E-7) in the wild-type yellow fever strain Senegal 1990, which also displayed a T153K mutation (Fig. 7). This virus strain was 5–10 times less sensitive to neutralization by the monoclonal antibody fragments compared to the other yellow fever strains tested (Fig. 3). Thus, the data can be reconciled by assuming that the antibodies bind to an epitope on the tip of the dimer, formed by amino acids from domains I and II of opposing chains (Fig. 7). Very similar to this finding, the conformational epitope of a chimpanzee derived monoclonal antibody which cross-neutralized dengue virus serotypes 1–4 and West Nile virus was recently shown to be defined by aa E-106 at the tip of domain II and E-137 in domain III of opposing E molecules (Goncalvez et al., 2004).

E-71 is the most critical amino acid of the identified YFV type-specific neutralizing epitope, because a mutation at this position conferred much higher resistance to neutralization with scFv-7A than mutations at E-153, E-154 or E-155 (95% vs. 25–50% in PRNT, respectively). A review of the literature for variability occurring in this region revealed that the Asn residue at position E-71 and Gln residue at position E-72 are 100% conserved among all wild-type and vaccine strains of YFV published to date, with the exception of one YFV strain isolated from mosquitoes in 1979 in Trinidad (Table 1). In contrast, the Thr at position E-153 and E-154 shows variation both in vaccine strains and in wild-type isolates (Table 1). A potential N-linked glycosylation site in the E protein of flaviviruses is located at aa E-151 to E-153, which is utilized by the 17DD and 17D-213 vaccine strains (Post et al., 1991), whereas neither of the wild-type strains of this study has a glycosylation site at this position (aa N-W-N/K at positions E-151 to E-153). Because the 17D-204-WHO vaccine strain used in this study displays N151, W152 and T153, the escape variants 7A-14 and 7A-18 could be glycosylation mutants (Table 1). The T154A mutation in virus variant 7A-14 has previously not been reported as a neutralization escape but was found in human wild-type YFV isolates from Ecuador and Peru (Table 1). Interestingly, the D155G mutation of our escape variant 7A-10 has been described together with a G303K

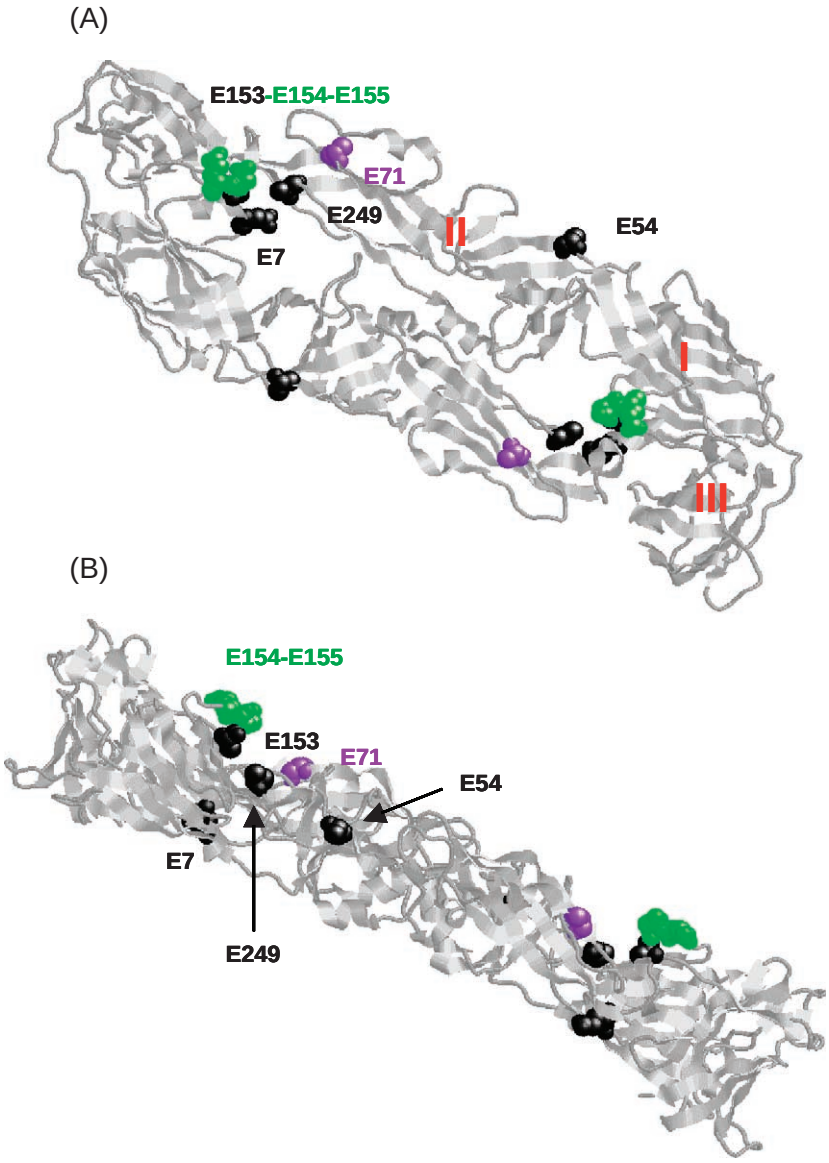

Fig. 7. Locations of residues E-71 and E-153, E-154, E-155 associated with neutralization escape, and naturally occurring mutations in the E protein of YFV strain Senegal 1990 (E-7, E-54, E-153 and E-249). The sequence of YFV-E was modeled on the 3D structure of the dengue virus type 2 envelope glycoprotein using RasMol (www.umass.edu/microbio/rasmol). (A) Top view of the E protein homodimer with domains I, II and III indicated. (B) Side view.

mutation in a 17D virus variant isolated from a human case of vaccine associated encephalitis (Jennings et al., 1994). The 17DD vaccine strains from Brazil and Senegal, which showed slightly increased neurovirulence in animal models (Barrett and Gould, 1986), both have a D155S substitution (Table 1).

The only neutralizing antibody specificity isolated from YF patients in this study was directed against one epitope located in domain II of the E protein of a YFV 17D vaccine virus. This is in line with observations that cross-reactive flavivirus antibodies are mostly directed against domain II, whereas strain-specific antibodies are directed against domain III of the envelope protein (Crill and Chang, 2004, Roehrig, 2003). However, it cannot be excluded that the antibody repertoire was restricted in the severely ill YF patients from which the libraries were derived. Whether we identified the major epitope by which 17D vaccine strains induce humoral immunity against wild-type YFV infection therefore needs to be tested in vivo in a relevant animal model.

We further investigated the capacity of polyclonal sera from yellow fever cases and immunized travelers to neutralize the two YFV-17D-derived escape variants with mutations N71K and D155G, respectively. The neutralizing titers of the YF patients were up to 10-fold reduced with the N71K variant, confirming the importance of E-71 for neutralization (Fig. 8). In contrast, the neutralizing titers of the vaccinees remained unchanged or were only slightly

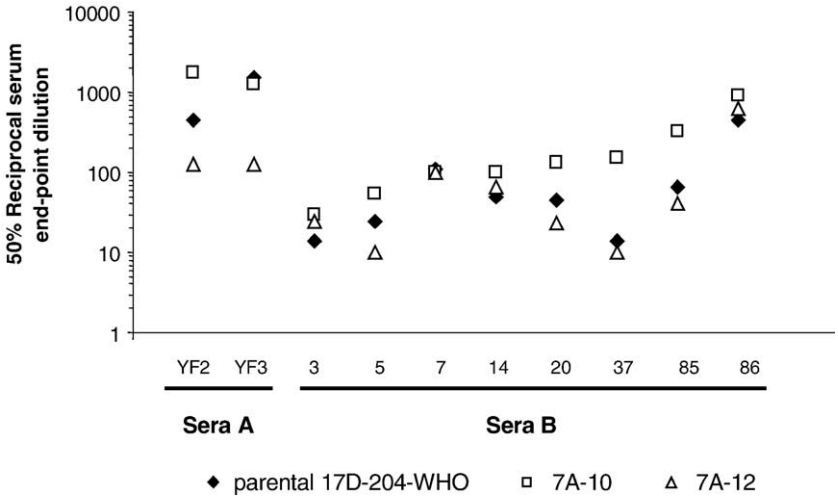

Fig. 8. Sensitivity of escape mutants 7A-10 and 7A-12 to neutralization by sera from YF patients (sera A) and from 17D vaccinees (sera B). As a positive control, the parental YFV-17D-204-WHO virus was used. 50% endpoint dilution represents the dilution of each serum at which 50% of the wells were protected from infection and is indicated as reciprocal dilution in a $\log_{10}$ scale. Each serum was tested in five replicates.

reduced. Paradoxically, the escape mutant 7A-10 (D155G, 50% neutralization resistance to the monoclonal antibody fragment) showed approximately 10-fold increased sensitivity to neutralization with half of the human sera. A possible explanation could be that the D155G mutation results in conformational changes in the envelope leading to exposure of other neutralizing epitopes.

Taken together, these in vitro data suggest that mutations in the E-71/E-153/E-154/E-155 epitope of wild-type or vaccine strains of YFV do not carry a risk of neutralization escape in persons immunized with the 17D vaccine.

Materials and methods

Virus strains

A commercially available preparation of the 17D-204-WHO yellow fever virus vaccine, manufactured by the Robert Koch Institute, Berlin, Germany, was used for the production of antigen and generation of neutralization escape variants. Wild-type yellow fever strains Asibi (Ghana 1927), ArD76320 (Senegal 1990), BA-55 (Nigeria 1987), ETH2777 (Ethiopia 1961) and HB1782 (CAR 1986) were obtained as lyophilized stocks made on suckling mouse brain; in addition, cell culture supernatant of a West Nile virus strain isolated in 2000 in France was used. Vero cells (ATCC-LGC) were grown in DMEM supplemented with 10% FCS, 2 mM glutamine and penicillin–streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were infected with a 1:10 dilution of the virus stocks and the supernatants were titrated on day 5 post-infection in a plaque assay. For virus neutralization assays, porcine kidney PS cells were grown in Leibovitz 15 medium (L-15) containing 5% FCS and penicillin–streptomycin at 37 °C in non-CO_2 humidified atmosphere.

Purification of YFV-17D, strain 204-WHO virions

Subconfluent Vero cells grown in T225 flask were infected with YFV-17D at a multiplicity of infection (moi) of 0.1 for 5 days at 37 °C. YFV-17D particles contained in the supernatant were purified by centrifugation at 70,000×*g* for 2 h at 4 °C through a 30% (v/v) glycerol/PBS cushion in a Beckmann SW28 rotor. Pelleted virions were resuspended in TNE buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) and proteins were quantified using the BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). Purity of the virus preparation was analyzed by SDS–PAGE.

Construction of antibody phage display libraries

Two phage libraries displaying single-chain antibodies (scFv) were constructed as previously described (Kontermann, 2001). Briefly, peripheral blood lymphocytes were prepared from two severely ill, but fully recovered yellow fever patients approximately 6 months after the acute infection. The patients had been identified during a yellow fever epidemic in 2000 in Guinea, West Africa (ter Meulen et al., 2004). Their YFV neutralizing antibody titers at the time of PBL donation were determined as 1:2560 by virus neutralization assay. Total RNA was extracted from $>10^7$ PBL (RNeasy kit, QIAGEN, Hilden, Germany) and mRNA was reverse transcribed into cDNA using random hexamer primers. Variable heavy and light chain genes were amplified from cDNA by a two-step nested PCR using a combination of 38 degenerated primers (Marks et al., 1991). The PCR-amplified κ and λ fragments were pooled, digested with *Apa*LI and *Not*I, and ligated to the phagemid vector pHEN3 (Korn et al., 2004). Electrocompetent TG1 *Escherichia coli* cells (Stratagene, La Jolla, CA, USA) were transformed with the ligation

products and expanded to obtain a variable κ light chain ($V_L\kappa$) and a variable λ light chain ($V_L\lambda$) pHEN3 sub-library, respectively. Phagemid DNA from these two sub-libraries was digested with *Sfi*I and *Xho*I and ligated to PCR-amplified variable heavy (V_H) fragments. The ligated products were electroporated into TG1 cells and expanded to obtain a (V_H-$V_L\kappa$)-pHEN3 and a (V_H-$V_L\lambda$)-pHEN3 library. These V_H-$V_L\kappa$ and V_H-$V_L\lambda$ libraries were infected with helper phages and the rescued recombinant phages were precipitated from cell culture supernatants with 20% PEG_{6000}/2.5 M NaCl and resuspended in PBS for subsequent panning.

Selection of YFV-binding antibody phage clones by panning

The two phage libraries were pooled and then panned with glycerol-purified YFV-17D particles essentially as described (Kontermann, 2001). Briefly, 10^{13} phage units from each library were panned in immunotubes (Maxisorp, Nunc, Wiesbaden, Germany) coated overnight with purified non-inactivated YFV-17D-204-WHO particles (25 μg/ml in 0.05 M carbonate buffer pH 9.6). Immunotubes were washed with PBS/0.1% Tween-20 and PBS. Bound phages were eluted with 0.1 M triethylamine and used to infect a fresh log-phase TG1 culture. For the identification of YFV-specific monoclonal phages, single TG1 colonies obtained from the third and the fourth round of selection were screened for binding. Bacteria grown in 96-well microtiter plates were infected with helper phages and grown overnight to allow the rescue of monoclonal phages. Supernatants containing monoclonal phages were tested in ELISA and phages bound to YFV were detected using an anti-M13 horseradish peroxidase-conjugated antibody (Pharmacia, Freiburg, Germany) diluted 1:5000. Diversity of clones was analyzed by BstN1 fingerprinting and DNA of single clones was sequenced with primers fdseq1 and LMB3 (Kontermann, 2001).

Production and purification of soluble scFvs

For soluble expression, scFv fragments were subcloned into the pAB1 plasmid. This vector introduces a His-tag in the C-terminus of the scFv and allows inducible expression in the periplasmic space of *E. coli* TG1 cells (Kontermann et al., 1997). ScFv fragments were purified from periplasmic preparations by immobilized metal affinity chromatography (IMAC) as described.

Single-chain antibody (scFv) ELISA

Microtiter wells were coated with glycerol-purified YFV-17D particles at a concentration of 25 μg/ml in 0.05 M carbonate buffer pH 9.6. After blocking with PBS/2% skimmed milk, the purified scFv were added and incubated for 1 h at room temperature. Bound scFvs were detected by incubation with a murine anti-His-tag antibody (Santa Cruz Biotechnology, Santa Cruz CA, USA) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dako, Hamburg, Germany), both diluted 1:1000. ABTS (KPL, Gaithersburg, USA), was used as substrate and absorbance was measured at 405 nm.

Production of radiolabeled YFV virions and immunoprecipitation (IP) assay

A T75 flask with subconfluent Vero cells was infected with YFV-17D-204-WHO (moi = 1). 24 h post-infection, the culture medium was removed and replaced with cysteine and methionine-free DMEM supplemented with 2% FCS, 2 mM glutamine and antibiotics for 1 h at 37 °C. Then, 1 mCi of [^{35}S] methionine and [^{35}S] cysteine (Promix Redivue, Amersham, Freiburg, Germany) was added. After 48 h the cell culture supernatant was harvested and radiolabeled virions were purified through a 30% glycerol cushion as described above. Pelleted virions were resuspended in TNE buffer containing the mild, non-ionic detergent 0.5% *n*-octyl-β-D-glucopyranoside (Sigma, Deisenhofen, Germany) and a protease inhibitor cocktail (Complete, Roche, Mannheim, Germany), and sonicated three times for 10 s at 4 °C. The preparation was cleared at 20,000 × *g* for 1 h at 4 °C, then 150 μl was incubated with 1.5 μg of scFv for 4 h at 4 °C. Antigen–scFv complexes were immunoprecipitated by addition of 1 μg anti-His-tag antibody and 20 μl protein G-coupled agarose beads (Santa Cruz Biotechnology, Santa Cruz CA, USA) overnight on an overhead rotator at 4 °C. IP was also performed with 1 μg each of YFV-E-specific mAbs 6538 and 6330, respectively, and 20 μl of protein G-coupled agarose beads. As negative control, IP was also performed with 1.5 μg of an irrelevant scFv or with anti-His-tag antibody alone. Samples were analyzed on a 12% SDS–PAGE gel exposed to X-ray film.

Biotinylation of purified scFv-7A and competition ELISA

The purified scFv-7A was biotinylated using the EZ-Link NHS-LC-Biotin kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions and dialyzed against PBS. 2 μg of biotinylated scFv-7A was mixed with increasing amounts of competitors (scFv or mAbs) and incubated in a 96-well plate coated with purified YFV-17D virions. Bound scFv-7A was detected using HRP-conjugated streptavidin (Pierce Biotechnology, Rockford, IL, USA) diluted 1:10000. Competition was expressed as the percentage of binding compared to biotinylated scFv-7A alone.

Virus titration and neutralization assays

The ability of the antibody fragments or of human sera to block YFV infection was tested in PRNT

using Vero or PS cells in a 6-well or 12-well format, or in a 96-well microneutralization assay using Vero cells.

(i) Virus titration using PS or Vero cells. Virus dilutions and trypsinized PS cells (4.8×10^5 cells/well) were added simultaneously to 12-well plates in a total volume of 1 ml/well and incubated for 3 h at 37 °C. The virus/cell mixture was overlaid with 1.6% carboxymethylcellulose in L-15 medium. After incubation for 4 days at 37 °C, cells were formalin fixed and stained with 500 ng/ml naphtol blue black. Virus was also titered on subconfluent Vero cells in 6-well plates. After an incubation of 1 h at 37 °C, the inoculum was removed, cells were washed once with PBS and overlaid with 2% low-melt agarose. On day 5 after infection plaques were visualized with 0.005% neutral red staining.

(ii) Plaque reduction neutralization assay (PRNT) using PS or Vero cells. Serial twofold dilutions of recombinant antibodies starting at a final concentration of 10 μg/ml were mixed with 100 plaque forming units (pfu) virus in a total volume of 1 ml. After an incubation period of 1 h at 37 °C, the virus/antibody mixture was seeded in 12-well plates together with PS cells and assayed for plaques as in (i). Human serum samples were inactivated for 30 min at 56 °C and used in serial twofold dilutions starting at 1:20. For PRNT on Vero cells, the antibody/virus mixture was incubated on subconfluent Vero cells and plaques were assayed as in (i). The percentage of neutralization was expressed as follows: 100 − [(number of plaques obtained with virus + scFv/number of plaques obtained with virus only) × 100].

(iii) Microneutralization assay with human sera. The 50% tissue culture infective dose ($TCID_{50}$) was determined for the parental YFV-17D-204-WHO strain and for the derived escape mutants 7A-10 and 7A-12. Each virus was serially 10-fold diluted in DMEM without FCS and 100 μl of each dilution (five replicates per dilution) was incubated for 1 h at 37 °C on 2.5×10^4 subconfluent Vero cells per well of a pre-seeded 96-well plate. The inoculum was removed and the cells were washed three times with PBS. 100 μl of DMEM with 2%FCS was added per well and the plate was incubated at 37 °C. After 6 days, the cytopathic effect was observed microscopically in each well and the $TCID_{50}$ was calculated using the Reed-Muench formula. Human serum samples from YF patients and 17D vaccinees were inactivated for 30 min at 56 °C and serially twofold diluted in DMEM without FCS starting at a dilution of 1:20. The 50% end-point dilution of each serum, corresponding to the dilution at which 50% of wells were completely protected from infection, was determined according to the Reed-Muench formula.

Selection of neutralization-resistant YFV-17D variants to scFv-7A

In order to map the neutralizing epitopes on the E protein, scFv-7A neutralization-resistant virus variants were generated and partially sequenced. 100 pfu of YFV-17D-204-WHO was incubated for 1 h at 37 °C with 95% of the scFv-7A concentration which had been shown to completely neutralize the inoculum (7 μg/ml, see Results section). The mixture was inoculated on Vero cells for 1 h at 37 °C in a 6-well plate format. The inoculum was removed and replaced with fresh medium supplemented with scFv-7A (7 μg/ml). Three days post-infection, 50 μl of the supernatant was incubated with 1 ml DMEM/scFv-7A (7 μg/ml) for 1 h at 37 °C and inoculated on cells for 1 h at 37 °C. After removing the inoculum, fresh medium containing scFv-7A (7 μg/ml) was added. This selection procedure was repeated twice. After the third round of selection, escape mutants contained in the supernatant were plaque purified. Twenty single clones were propagated, plaque-titered and tested in PRNT with various concentrations of scFv-7A as described above.

Sequencing of prM and E proteins

Viral RNA was isolated from infected cell culture supernatants using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). The E protein of the neutralization escape mutants was amplified by RT/PCR and sequenced as previously described (ter Meulen et al., 2004). RT-PCR and sequencing of the M protein were performed using a prM forward primer (5′-gatgttctgactgtgcaatt-3′) and an M reverse primer (5′-ttcctccatgcacccct-3′).

Wild-type YFV strains were first genotyped by amplifying a 670 nucleotide fragment within the prM-E region (Mutebi et al., 2001). The remainder of the E protein was sequenced using two sets of primers that generate two overlapping fragments. Primers were designed aligning wild-type YFV sequences accessible in the GenBank and designated EnvF1 (5′ tgtgaagattaatgacaagtgcc), EnvF2 (5′ caatgataagtgcccgagc), EnvF3 (caggtcatggcacggttg), EnvR1 (ggattgacttcaattaggacttc), EnvR2 (5′ cacctcaatcagcacttcatc), NS1R1 (5′ cactattgatgcaagcttcacag) and NS1R2 (5′ tctccacatttgagctctcg). Due to the sequence variability of the envelope protein gene, the East/Central African strains were amplified using primer combinations EnvF2/EnvR1 and EnvF3/NS1R2, and the West African strains using EnvF1/EnvR2 and EnvF3/NS1R1.

Acknowledgments

This work was supported by the Howard Hughes Medical Institute, Infectious Disease and Parasitology Program, grant #55000639, the European Community, INCO-DEV grant ICA4-CT2002-10050 and the Kempkes Stiftung, Marburg. We thank the team of the Viral

Hemorrhagic Fever Project Guinea (PFHG) for working with patients, and M. Niedrig, Robert Koch Institute Berlin, for providing the mouse monoclonal antibodies.

References

Barrett, A.D.T., Gould, E.A., 1986. Comparison of neurovirulence of different strains of yellow fever virus in mice. J. Gen. Virol. 67, 631–637.

Brandriss, M.W., Schlesinger, J.J., Walsh, E.E., Briselli, M., 1986. Lethal 17D yellow fever encephalitis in mice: I. Passive protection by monoclonal antibodies to the envelope proteins of 17D yellow fever and dengue 2 viruses. J. Gen. Virol. 67, 229–234.

Crill, W.D., Chang, G.J., 2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J. Virol. 78 (24), 13975–13986.

Davies, R., Padlan, E.A., 1990. Antibody-antigen complexes. Annu. Rev. Biochem. 59, 439–473.

Dunster, L.M., Wang, H., Ryman, K.D., Miller, B.R., Watowich, S.J., Minor, P.D., Barrett, A.D., 1999. Molecular and biological changes associated with HeLa cell attenuation of wild-type yellow fever virus. Virology 261, 309–318.

Gelderblom, H.R., Kocks, C., L'Age-Stehr, J., Reupke, H., 1985. Comparative immunoelectron microscopy with monoclonal antibodies on yellow fever virus-infected cells: pre-embedding labelling versus immunocryo-ultramicrotomy. J. Virol. Methods 10, 225–239.

Goncalvez, A.P., Purcell, R.H., Lai, C.-J., 2004. Epitope determinants of a chimpanzee Fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to the fusion loop of the dengue type 2 virus envelope glycoprotein. J. Virol. 78, 12919–12928.

Gould, E.A., Buckley, A., Barrett, A.D.T., Cammack, N., 1986. Neutralizing (54K) and nonneutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. J. Gen. Virol. 67, 591–595.

Jennings, A.D., Whitby, J.E., Minor, P.D., Barrett, A.D., 1993. Comparison of the nucleotide and deduced amino acid sequences of the structural protein genes of the yellow fever 17DD vaccine strain from Senegal with those of other yellow fever vaccine viruses. Vaccine 11, 679–681.

Jennings, A.D., Gibson, C.A., Miller, B.R., Mathews, J.H., Mitchell, C.J., Roehrig, J.T., Wood, D.J., Taffs, F., Sil, B.K., Whitby, S.N., 1994. Analysis of a yellow fever virus isolated from a fatal case of vaccine-associated human encephalitis. J. Infect. Dis. 169, 512–518.

Kitchener, S., 2004. Viscerotropic and neurotropic disease following vaccination with the 17D yellow fever vaccine, ARILVAX. Vaccine 22, 2103–2105.

Kontermann, R.E., 2001. In: Kontermann, R.E., Dübel, S. (Eds.), Antibody Engineering. Springer, Heidelberg, pp. 137–148.

Kontermann, R.E., Martineau, P., Cummings, C.E., Karpas, A., Allen, D., Derbbyshire, E., Winter, G., 1997. Enzyme immunoassays using bispecific diabodies. Immunotechnology 3, 137–144.

Korn, T., Nettelbeck, D.M., Volkel, T., Muller, R., Kontermann, R.E., 2004. Recombinant bispecific antibodies for the targeting of adenoviruses to CEA-expressing tumour cells: a comparative analysis of bacterially expressed single-chain diabody and tandem scFv. J. Gene Med. 6, 642–651.

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S., Strauss, J.H., 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108, 717–725.

Lawrence, G.L., Burgess, M.A., Kass, R.B., 2004. Age-related risk of adverse events following yellow fever vaccination in Australia. Commun. Dis. Intell. 28, 244–248.

Lindenbach, B.D., Rice, C.M., 2001. Flaviviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. I. Lippincott, Williams and Wilkins, Philadelphia, pp. 991–1041.

Lobigs, M., Dalgarno, L., Schlesiger, J.J., Weir, R.C., 1987. Location of a neutralization determinant in the E protein of yellow fever virus (17D vaccine strain). Virology 161, 474–478.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G., 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. 222, 581–597.

Martin, M., Tsai, T.F., Cropp, B., Chang, G.J., Holmes, D.A., Tseng, J., Shieh, W., Zaki, S.R., Al-Sanouri, I., Cutrona, A.F., Ray, G., Weld, L.H., Cetron, M.S., 2001. Fever and multisystem organ failure associated with 17D-204 yellow fever vaccination: a report of four cases. Lancet 358, 98–104.

McArthur, M.A., Suderman, M.T., Mutebi, J.P., Xiao, S.Y., Barrett, A.D., 2003. Molecular characterization of a hamster viscerotropic strain of yellow fever virus. J. Virol. 77, 1462–1468.

Monath, T.P., 2004. Yellow fever vaccine. In: Plotkin, S.A., Orenstein, W.A. (Eds.), Vaccines. Saunders, Philadelphia, USA, pp. 1095–1176.

Monath, T.P., Barrett, A.D.T., 2003. Pathogenesis and pathophysiology of yellow fever. Adv. Virus Res. 60, 343–395.

Mutebi, J.P., Wang, H.A., Li, L., Bryant, A.D., 2001. Phylogenetic and evolutionary relationships among yellow fever virus isolates in Africa. J. Virol. 75, 6999–7008.

Pincus, S., Mason, P.W., Konishi, E., Fonseca, B.A., Shope, R.E., Rice, C.M., Paoletti, E., 1992. Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever virus encephalitis. Virology 187, 290–297.

Post, P.R., Carvalho, R., Galler, R., 1991. Glycosylation and secretion of yellow fever virus nonstructural protein NS1. Virus Res. 18, 291–302.

Pugachev, K.V., Ocran, S.W., Guirakhoo, F., Furby, D., Monath, T.P., 2002. Heterogeneous nature of the genome of the ARILVAX yellow fever 17D vaccine revealed by consensus sequencing. Vaccine 20, 996–999.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from the tick-borne encephalitis virus at 2 Å resolution. Nature 375, 291–298.

Robertson, S.E., Hull, B.P., Tomori, O., Bele, O., LeDuc, J.W., Esteves, K., 1996. Yellow fever: a decade of re-emergence. J. Am. Med. Assoc. 276, 1157–1162.

Roehrig, J.T., 2003. Antigenic structure of flavivirus proteins. Adv. Virus Res. 59, 141–175.

Ryman, K.D., Ledger, T.N., Weir, R.C., Schlesinger, J.J., Barett, A.D.T., 1997a. Yellow fever virus envelope protein has two discrete type-specific neutralizing epitopes. J. Gen. Virol. 78, 1352–1356.

Ryman, K.D., Xie, H., Ledger, T.N., Campbell, G.A., Barrett, A.D.T., 1997b. Antigenic variants of yellow fever virus with an altered neurovirulence phenotype in mice. Virology 230, 376–380.

Ryman, K.D., Ledger, T.N., Campbell, G.A., Watowich, S.J., Barrett, A.D.T., 1998. Mutations in a 17D-204 vaccine substrain-specific envelope protein epitope alter the pathogenesis of yellow fever virus in mice. Virology 244, 59–65.

ter Meulen, J., Sakho, M., Koulemou, K., Magassouba, N., Bah, A., Preiser, W., Daffis, S., Klewitz, C., Bae, H.G., Niedrig, M., Zeller, H., Heinzel-Gutenbrunner, M., Koivogui, L., Kaufmann, A., 2004. Activation of the cytokine network is related to unfavourable outcome in Yellow fever. J. Infect. Dis. 190, 1821–1827.